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Whole spermatangia within the seminal receptacles of female chokka squid (*Loligo reynaudii* Orbigny, 1845)

NORIYOSI SATO[1], YOKO IWATA[2], PAUL W. SHAW[3,4], WARWICK H. H. SAUER[4]

[1]*Oki Marine Biological Station, Shimane University, Oki, Shimane, 6850024, Japan.*
[2]*Atmosphere and Ocean Research Institute, University of Tokyo, Kashiwa, Chiba 2778564, Japan.*
[3]*Institute of Biological, Environmental and Rural Science (IBERS), Aberystwyth University, Penglais, Aberystwyth SY23 3DA, UK*
[4]*Department of Ichthyology and Fisheries Science, Rhodes University, PO Box 94, 6140 Grahamstown, South Africa.*

Author for correspondence:
Noriyosi Sato
E-mail address: norico3000@gmail.com

During cephalopod copulation, sperm are transferred from males to females through a complex process (Hanlon & Messenger, 2018) involving males depositing sperm packets (spermatophores) onto various locations on or inside the female (Hanlon & Messenger, 2018). When spermatophores are released from the Needham's sac through the male funnel, the cap thread of the spermatophore is pulled and triggers a spermatophoric reaction (Drew, 1919; Marian, 2012, 2015; Sato *et al*., 2013; Sato, Kasugai & Munehara, 2014). Through this reaction, a sac containing a sperm mass (spermatangium) is ejaculated from the spermatophore and attaches to the female by a cement body and numerous small stellate particles (Drew, 1919; Austin, Lutwak-Mann & Mann, 1964; Takahama *et al*., 1991; Marian, 2012; Sato *et al*., 2014). Females of many species of squid and cuttlefish have sperm storage organs (seminal receptacles) within the buccal membrane, and spermatangia are generally attached on and around the buccal mass (e.g. in Idiosepidae (Sato *et al*., 2010), Loliginidae (Drew, 1911; van Oordt 1938; Lum-Kong, 1992; Wada *et al*., 2005; Iwata & Sakurai, 2007: Saad *et al*., 2018), and Sepiidae (Hanlon, Ament & Gabr, 1999; Naud *et al*., 2005)). How sperm is transfer from the spermatangium to the seminal receptacle, however, is poorly understood. Spermatozoa are ejaculated from the distal tip of spermatangia after completion of the spermatophoric reaction, but the spermatozoa are not directly transferred to the seminal receptacle through this ejaculation because the distal tip does not connect directly to the opening of the seminal receptacle (Drew, 1919; Iwata *et al*., 2011; Marian, 2012, 2015; Sato *et al*., 2014; Fernández-Álvarez *et al*., 2018).

Previous studies have offered several hypotheses to explain sperm transfer in squid, including sperm actively swimming, muscular action of the seminal receptacle and female manipulation. The most intuitive hypothesis is that spermatozoa ejaculated from the spermatangium actively swim into the seminal receptacle. Spermatozoa become active on contact with seawater (Drew, 1919; van Oordt, 1938; Marian, 2012; Sato *et al*., 2014), whilst they become immobilized once inside the seminal receptacle (van Oordt, 1938), where their heads align facing the internal epithelium (Drew, 1911; van Oordt, 1938; Sato *et al*., 2010; Fernández-Álvarez *et al*., 2018), suggesting active swimming towards sperm-attracting substances. A recent microscopy study demonstrated free sperm within the opening to the seminal receptacle in *Dosidicus gigas* (Fernández-Álvarez *et al*., 2018), and that the spermatozoa were the only cellular component of the seminal solution (mobile spermatozoa and immobile round cells) found inside the seminal receptacle, reinforcing the active swimming hypothesis. It is not known how spermatozoa might be attracted to the seminal receptacle, but sperm-attracting peptides are present in the eggs of *Sepia* (Zatylny *et al*., 2002) and

Octopus (De Lisa *et al*., 2013).

Another hypothesis is that females may transfer whole spermatangia directly into the seminal receptacle using their arms. Caribbean reef squid have been suggested to employ this method (Moynihan & Arcadio, 1982; Hanlon & Messenger, 2018), but these studies were based on direct behavioural observation in the field and lacked histological confirmation of the spermatangia in the receptacle. Van Oordt (1938) observed whole spermatangia inside the seminal receptacle in *Loligo vulgaris*, and used this observation to forward the hypothesis of muscular suction generated by the seminal receptacle. Manipulation of attached spermatangia by the female has been reported in *Idiosepius paradoxus* (Sato *et al*., 2013) and in *Sepiadarium austrinum* (Wegener et al., 2013), but these cases were associated with removal or ingestion, rather than with sperm transfer.

With the exception of van Oordt (1938) and Saad et al.(2018), most previous studies involving multiple cephalopod species have not reported whole spermatangia within the seminal receptacle (e.g. Drew, 1911; Lum-Kong, 1992; Hanlon *et al*., 1999; Naud *et al*., 2005; Sato *et al*., 2010, Bush *et al*., 2012; Fernández-Álvarez *et al*., 2018).

In the present study, we report finding whole spermatangia inside the seminal receptacle of another loliginid, the chokka squid *Loligo reynaudii*. Based on this finding, we further explore the possibilities of sperm transfer mechanisms in squids.

Ten female squid (dorsal mantle length 177 to 228 mm) were collected by jigging, on the 16 November 2008 and 11 September 2009 in St Francis Bay, South Africa. All females were mature according to the maturity scale of Perez, Aguiar & Oliveira (2002) for *Doryteuthis plei*. The seminal receptacle was dissected from each individual and fixed in Bouin's solution, then dehydrated and embedded in paraffin wax. Serial 8–10µm sections were made in transverse (5 individuals) and longitudinal (5 individuals) orientations, and stained with hematoxylin and eosin using standard methods. The largest diameter of the central duct and the greatest depth of the seminal receptacle were measured at the mid-sagittal section, from photographs using ImageJ software (NIH, Bethesda, MD, USA).

The duct was found to run through the centre of the seminal receptacle and to branch into a number (14-37) of small bulb-shaped compartments (bulbs) (Fig. 1). The mean diameter of the duct was 270µm (n=5, range 99–464µm) and depth of the seminal receptacle was 2166µm (n=5, range 1797–2384µm) (Fig. 1A). Columnar ciliated epithelium lined the duct (Fig. 1B), cuboidal ciliated epithelium cells lined the bottom of each bulb (Fig. 1C), and goblet cells were distributed in the neck of each bulb (Fig. 1C). The seminal receptacles were surrounded by a muscle sheath and connective tissue, and individual bulbs were separated by a muscle sheath (Fig. 1C). In 75 of 198 bulbs observed sperm were aligned with their heads facing the epithelium (Fig 1D), whereas in the remaining bulbs sperm were not aligned but rather, were distributed randomly, similar to sperm located near the bulb entrance or in the central duct (Fig. 1A, B).

In all females, at least ten intact spermatangia (containing sperm) were observed attached to the external surface of their buccal mass near the seminal receptacle (Fig. 2A). Whole spermatangia structures (cement body + tunic containing varying amounts of sperm) were found within the seminal receptacles of five of the ten examined females (Fig. 2B). In these latter spermatangia, the cement bodies were attached to, and interspersed with, the epithelium of the seminal receptacle (Fig. 2B, C). One receptacle contained two spermatangia (width 375µm and 354µm) which were filled with spermatozoa (Fig. 2B), and the whole spermatangial structure (tunic, sperm mass and cement body) appeared identical to spermatangia attached to the outside of the buccal mass, except for their tips which appeared partly shrivelled. In this seminal receptacle, spermatozoa near the tip of the intact spermatangia were not aligned towards the bulb walls (Fig. 2C). The remaining four receptacles each contained only one spermatangium, which in all cases contained few spermatozoa within an inner tunic (Fig. 2D). In three of these latter four cases, the inner tunic was collapsed (Fig. 2E) or missing, and only the cement body remained (Fig. 2F).

Male *L. reynaudii* employ two distinct mating tactics: large consort males mate in a “male-parallel” position and attach spermatophores near the oviduct opening, while small sneaker males mate in a “head-to-head” position and attach spermatophores near the seminal receptacle (Hanlon, Smale & Sauer, 2002). Dimorphism in spermatangium size and morphology has also been demonstrated between large consort and small sneaker males in this species, taking “rope-like” and “drop-like” forms respectively (Iwata *et al*., 2018). Spermatangia in and around the seminal receptacle were found in the present study to be of the drop-like form only, and therefore assumed to be deposited by sneaker males.

As well as free individual spermatozoa present within the seminal receptacles, as expected from previous studies of squid and cuttlefish (Drew, 1911; van Oordt, 1938; Lum-Kong, 1992; Hanlon, *et al*. 1999; Naud, *et al*., 2005; Sato *et al*., 2010; Bush *et al*., 2012; Fernández-Álvarez *et al*., 2018), the present study also found whole spermatangia within the seminal receptacle of 50% of females examined. This result prompts the question as to how these spermatangia were transferred into the seminal receptacle? After copulation, the female may use her arms to transfer whole spermatangia into the seminal receptacle, with the spermatangia taken from those attached around the buccal mass. Manipulation of deposited spermatangia has been observed in pygmy squid, *Idiosepius paradoxus*, where females remove some of the attached spermatangia after mating (Sato *et al*., 2013; Sato, Yoshida & Kasugai, 2017). Likewise in *Sepia apama* the number of male genotypes represented by the sperm within the seminal receptacle is less than those represented in spermatangia attached around the buccal mass, suggesting that the female may control sperm or spermatangia transfer into the seminal receptacle (Naud *et al*., 2005). If females physically transfer the spermatangia, however, the cement body would be expected to remain attached to the external surface of the buccal mass and not anchored to the epithelium of the seminal receptacle, whereas we observed that the cement body was intact and strongly attached to the epithelium within the seminal receptacle. The appearance of the connection of the cement substance to the epithelium of the seminal receptacle described here is similar to that observed between the cement substance of the attached spermatangia and the connective tissue in *Doryteuthis plei* (Marian, 2012). The presence of a strong attachment of the cement body to the epithelium of the receptacle in *L. reynaudii* suggests that the spermatophores are implanted into the organ whilst the spermatophoric reaction is occurring (i.e. during mating) rather than post-mating translocation by the female. Therefore, we also hypothesize that once inside the receptacle, sperm is released from the tip of the spermatangium.

The presence of spermatangia within the seminal receptacle may also suggest a different process of sperm storage and usage during fertilization. Spermatozoa are assumed to be released and swim freely from the tip of the spermatangia towards eggs or the receptacle, but in the case of spermatangia transferred directly within the receptacle, their sperm may be released by being "squeezed" out by pressure from the muscles of the seminal receptacle. Most of the spermatangia observed here inside the receptacle displayed a "squashed" shape and collapsed inner tunic. In cephalopods, released sperm become active on contact with seawater (e.g. Sato et al 2014). However, sperm in the seminal receptacle are of course not released directly into seawater, and they may remain inactive. This transfer method may therefore result in sperm not being aligned towards the bulb epithelium. Half of the females examined had spermatangia inside the seminal receptacle, suggesting that this phenomenon may be common in this species. Interestingly, sperm not facing the bulb epithelium were observed in all samples, irrespective of whether whole spermatangia were located within the seminal receptacle. We note that sperm not facing the bulb epithelium in some bulbs of the seminal receptacle, is also encountered in other loliginid species (*L. pealii*, Drew, 1911; *L. vulgaris*, van Oordt, 1938; *L. forbesi*, Lum-Kong, 1992), as well as in other cephalopod groups (*S. officinalis*, Hanlon *et al*., 1999; *Bathyteuthis berryi*, Bush *et al*., 2012). This is not the case for all cephalopods however: *I. paradoxus* (Sato *et al*., 2010), *Octopus vulgaris* (Cuccu *et al*., 2013), *O. mimus* (Olivares *et al*., 2017) and *Dosidicus gigas* (Fernández-Álvarez *et al*., 2018) all were found to show stored sperm facing the bulb epithelium. Recently, Saad et al. (2018) reported intact spermatangia stored in the seminal receptacle in *D. plei*. They hypothesized those spermatangia serve as mating plugs and not for sperm storage, as most of them are concentrated in the opening duct of the seminal receptacle with the tips protruding outside the receptacle. This phenomenon was not observed in the present study, but rather the whole spermatangia here were situated well within the bulbs rather than in the duct openings.

In conclusion, our study observed whole spermatangia within the seminal receptacle of 50% of female squid examined, demonstrating that sperm storage by direct transfer of spermatangia may occur frequently in *L. reynaudii*, in addition to active migration of sperm over the buccal membrane and into the seminal receptacle from externally stored spermatangia. Our results do not preclude active swimming by individual sperm, given that most females had many spermatangia attached around the external surface of the seminal receptacle, and sperm stored in some bulbs were aligned facing towards the epithelium, suggesting that these sperm might have entered the storage organ through active swimming. In addition, it has been established in *H. bleekeri* that the flagellum of sneaker sperm, from the spermatangia attached around the seminal receptacle, is longer than that of consort sperm, from spermatangia placed around the oviduct (Iwata *et al*., 2011). There are no differences in swimming velocity between consort and sneaker sperm (Iwata *et al*., 2011), but sneaker sperm form clusters (Hirohashi *et al*., 2013) and display asymmetrical movement, using their long flagella, while moving along CO_2 gradients (Iida *et al*., 2017), behaviours proposed to be adapted to reach the seminal receptacle through active swimming (Hirohashi *et al*., 2016). *Loligo reynaudii* has a similar reproductive strategy to that of *H. bleekeri*, with morphological dimorphism in spermatangia between consort and sneaker males (Iwata *et al*., 2018). Sperm transfer and storage in *L. reynaudii* (Fig. 1B, D) may therefore be conducted by a combination of active swimming of sperm ejaculated from external spermatangia and by direct transfer of spermatangia into the seminal receptacle. However, additional research is required to confirm this.

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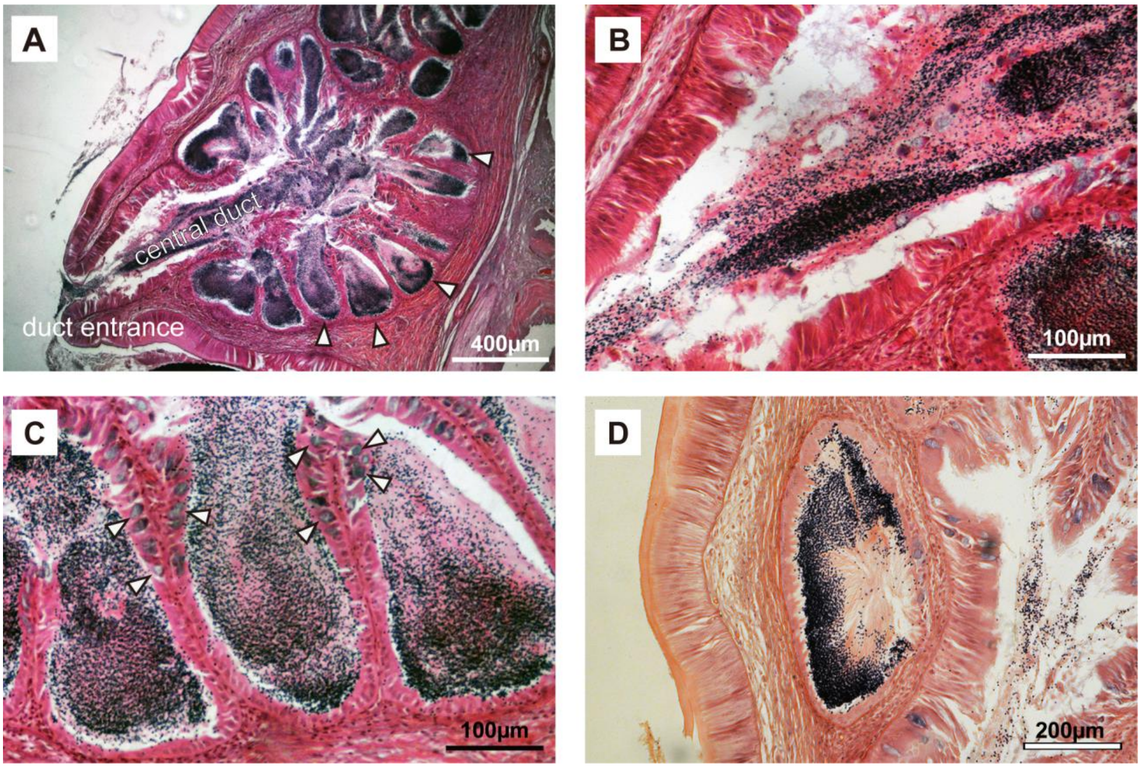

Figure 1. Structure of the seminal receptacle in *Loligo reynaudii*. **A.** Entire receptacle in longitudinal section, showing the external opening, central duct and peripheral bulbs (arrowhead indicates a peripheral bulb). **B.** Spermatozoa located in the central duct of the seminal receptacle. **C.** Longitudinal section of individual bulbs, with part of the sperm with the heads not facing the epithelium (arrowhead indicates goblet cells). **D.** Bulbs filled with sperm facing to the epithelium.

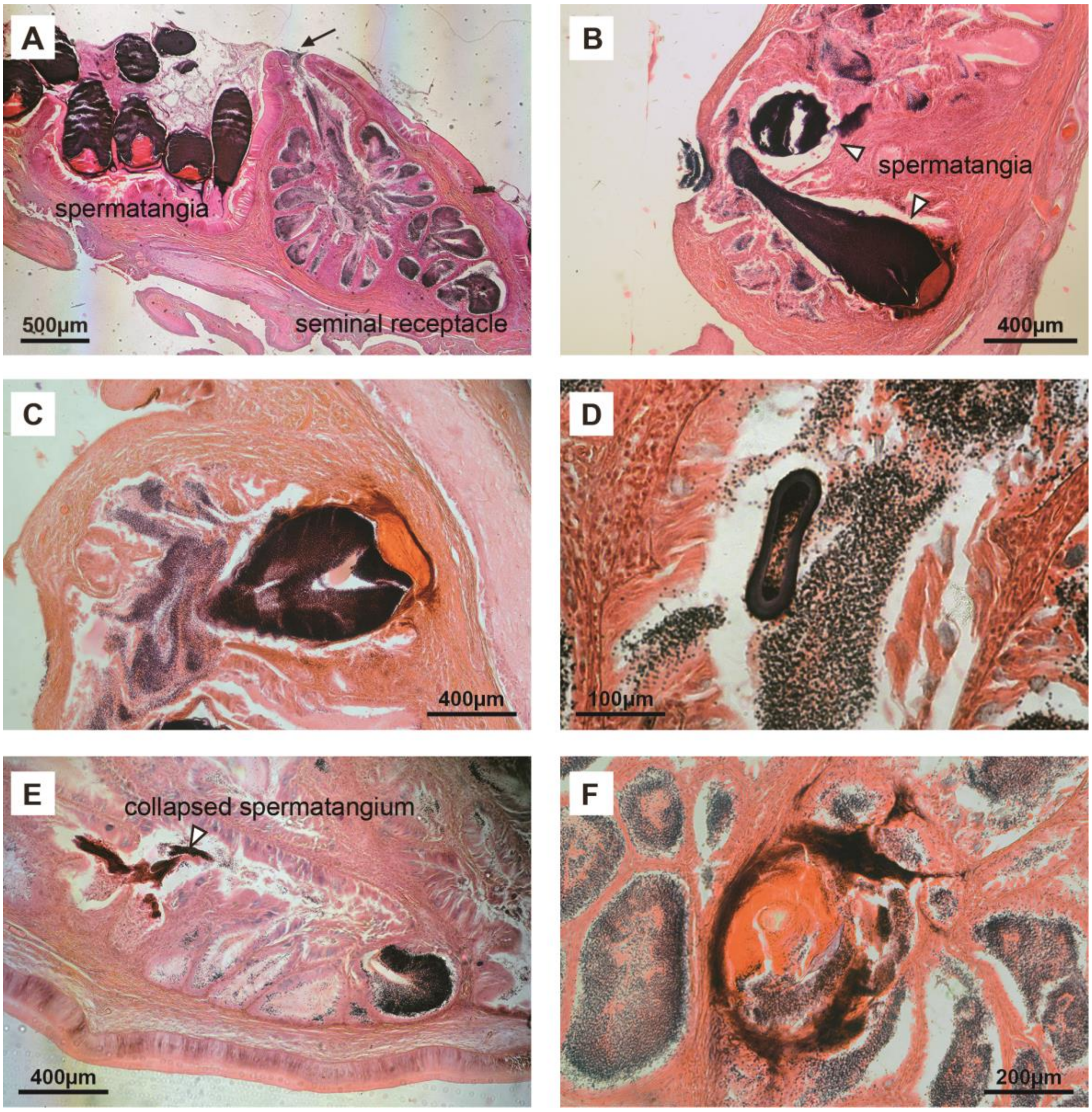

Figure 2. Spermatangia attached around and within the seminal receptacle. **A.** Multiple spermatangia (to the left) attached to the buccal membrane around the seminal receptacle (to the right) (black arrow indicates the opening of the seminal receptacle). **B.** Two spermatangia stored within the seminal receptacle. **C.** A spermatangium and released spermatozoa within the seminal receptacle. **D.** A spermatangium containing a small volume of spermatozoa. **E.** Collapsed spermatangium in the seminal receptacle

containing a small volume of sperm, and most bulbs showing few sperm but one bulb containing many sperm (to right). **F.** Transverse section of the cement body in the seminal receptacle.